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Temporal and spatial expression of matrix metalloproteinases during wound healing of human corneal tissue

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Abstract

Our understanding of MMP expression during corneal repair has previously relied upon animal models, isolated human biopsy specimens and cell culture studies. The aim of this study was to determine the temporal and spatial expression of matrix metalloproteinases following wounding of cultured human corneal tissue. Human corneas were cultured and cut into six pieces. The epithelium was removed with a corneal brush. The tissue was then re-cultured and tissue pieces were fixed up to 7 days post-wounding. Matrix metalloproteinases were detected by in situ hybridisation and immunohistochemistry. Intracellular laminin-5, a marker of migratory epithelial cells, was located immunohistologically. In the time scale studied tissue series from nine corneas achieved coverage of the stroma with epithelial cells and partial repair of damaged basement membrane, demonstrated by the Periodic acid-Schiff reaction and haematoxylin and eosin counter-staining. By day 3, migrating epithelial cells and stromal cells beneath the wounded area expressed collagenase-1 (MMP-1). Stromelysin-1 (MMP-3) was expressed only by fibroblast-like stromal cells. Stromelysin-2 (MMP-10) was detected in migrating epithelial cells and remained when the stroma was surrounded by a monolayer of epithelial cells. By day 7, development of multi-layered epithelium around the tissue coincided with cessation of MMP expression in both epithelial and stromal cells, except for MMP-9, which remained in epithelial basal cells. Tissue inhibitor of matrix metalloproteinase-1 was mainly associated with stromal cells and was reduced upon formation of a multi-layered epithelium. This study demonstrates matrix metalloproteinase expression in epithelial and fibroblast-like cells following wounding of human corneal tissue in culture where the cells remain in contact with their natural matrices.

Keywords: corneal wound healing; metalloproteinase; temporal modulation; laminin-5; TIMP-1; gelatinase

1. Introduction

The process of wound healing determines functionality of the repaired tissue. Understanding the sequence of events involved in the eye is particularly important as the tissue repair process determines the quality of vision post-insult.

Immediately following injury to the cornea keratocytes in the stroma at the periphery of the wound undergo apoptosis and re-epithelialisation begins (Wilson et al., 1996; Mohan et al., 1997). At the wound edge epithelial cells increase intracellular protein synthesis and re-organise their actin cytoskeleton (Gipson and Anderson, 1977), hemidesmosomes between basal cells and basement membrane (BM) are lost and superficial cell sloughing causes thinning of the epithelium to a single cell layer with formation of filopodia and lamellipodia (Pfister, 1975; Brewitt, 1979; Crosson et al., 1986). Extent of injury to the BM appears to play an important role in re-epithelialisation. In a mouse model of corneal debridement it was found that loss of basement membrane molecules and ultrastructure correlated with wounds that took longer than 24 hr to heal (Sta Iglesia and Stepp, 2000). Epithelial cell migration, mostly as a single cell layer, progresses independently of

Abbreviations: LN-5, laminin-5; BM, basement membrane; BWL, Bowman's layer.

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proliferation until wound closure. Epithelial thickness is restored by proliferation and upward movement of cells from the basal layer (Hanna and O'Brien, 1996). These transit cells are supplied by division of stem cells residing in the limbus at the periphery of the cornea (Dua and Azuara-Blanco, 2000). Secure attachment of the epithelium to the basement membrane is provided by new anchoring fibrils and hemidesmosomes. This process may be slowed if the migrating cells are also required to secrete new BM (Hirst et al., 1981).

As epithelial cells re-surface the wound, fibroblasts (activated keratocytes) migrate into the wound and proliferate. The fibroblasts reach maximum density in the stroma as the overlying epithelium differentiates into a mature multi-layered structure (Moller Pedersen et al., 1998). They produce new extracellular matrix and re-modelling can continue for several months.

The matrix metalloproteinases (MMPs) are a group of extracellular matrix degrading enzymes (Birkedal-Hansen, 1995). The MMP family currently includes more than 25 members, which can be divided into collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -7, -10), matrilysins (MMP-7 and -26), membrane-type MMPs (MMP-14, -15, -16, -17 and -24) and other MMPs according to their structure and substrate specificity (Uria and Lopez-Otin, 2000; Visse and Nagase, 2003). MMPs are secreted as inactive proenzymes that are activated outside the cell. Among other factors, the tissue inhibitors of matrix metalloproteinases (TIMPs) regulate MMP activity. The MMP:TIMP ratio assists in the regulation of the overall increase/decrease in matrix degradation and delayed wound healing may result from its imbalance (Vaalamo et al., 1999).

A study of the temporal expression of MMPs during cutaneous healing in a mouse model demonstrated the involvement of several MMPs throughout re-epithelialisation and stromal repair (Madlener et al., 1998). Biopsies and tear fluid samples from human corneas have demonstrated elevated MMP levels in various pathological conditions (Geerling et al., 1998; Zhou et al., 1998; Alfonso et al., 1999 p. 3; Smith et al., 1999; Garrana et al., 1999; Sobrin et al., 2000). However, our understanding of the profile of MMP expression throughout normal wound healing in the cornea has so far relied also upon animal studies (Azar et al., 1996; Maeda et al., 1998; Fini et al., 1998; Sivak and Fini, 2002).

To date evidence for the expression of MMPs in the cornea has been provided by isolated human biopsy specimens, often from pathological tissue, or by animal models. It is not possible to repeatedly biopsy the human corneas to determine MMP profiles during healing as the risk of long-term damage and vision loss due to scarring is very high. For this reason wounded and cultured human corneal tissue was used to study the temporal and spatial expression of MMPs by cells residing on and in their normal matrix environments during wound healing.

2. Materials and methods

2.1. Corneal tissue

Whole human corneas or the outer limbal rims remaining after corneal transplantation, from Moorfields Eye Hospital NHS Trust Eye Bank, UK, were stored at 4°C in Optisol (Means et al., 1995). All tissue was accompanied by informed research consent and the study followed the tenets of the Declaration of Helsinki. Within 48 hr (whole corneas) or within 7 days (limbal rims) post-mortem, tissues were cultured and wounded as previously described (Daniels et al., 2003b). Corneal tissue was incubated for 4 days at 37°C in 5% CO_2 in air with keratinocyte culture medium (KCM) to allow re-growth of any epithelium lost from the corneal tissue during surgical manipulation. KCM consisted of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 in a 1:1 ratio (Gibco Life Technologies, Paisley, Scotland, UK). KCM was supplemented with 10% (v/v) foetal calf serum (Gibco Life Technologies), 2 mM L-glutamine, 25 ng ml^{-1} adenine, 0·4 μg ml^{-1} hydrocortisone, 5 μg ml^{-1} insulin, 8·5 ng ml^{-1} cholera toxin, 140 ng ml^{-1} triiodothryonine, 5 μg ml^{-1} transferrin, 4 ng ml^{-1} epidermal growth factor, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (all Sigma Chemical Company, Poole, Dorset, UK).

2.2. Tissue wounding

Each cornea was cut into six equal pieces using a scalpel. One piece was left unwounded to confirm the presence of epithelium on the original sample. The remaining tissues were wounded using a rotating battery operated corneal brush (Algerbrush II, Algerbrush Company Inc., Lago Vista, TX, USA). The brush was moved along the tissue from the outside edge, avoiding the limbus where the epithelial stem cells reside, towards the centre until all of the epithelium from the starting point was removed (Fig. 1). Four of the wounded tissue pieces were cultured, free-floating, in KCM for up to 7 days. At intervals, including immediately post-wounding, tissue was fixed in 4% paraformaldehyde at room temperature overnight, dehydrated through a series of alcohols and embedded in paraffin. Tissue sections (5 μm), were cut onto Superfrost Plus microscope slides (BDH Laboratory Supplies, Poole, Dorset, UK).

2.3. Histology

The periodic-acid Schiff (PAS) reaction was performed to detect the presence of corneal epithelial basement membrane. Tissue sections were re-hydrated to distilled water and then treated with 1% periodic acid solution for 10 min. The sections were rinsed well in distilled water and then treated with Schiff's reagent for up to 20 min. Following washing under tap water for 5–10 min the nuclei

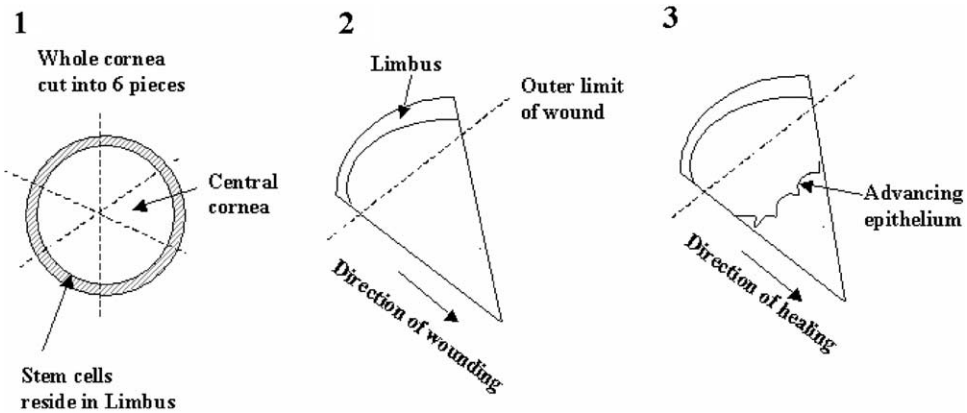


Fig. 1. Whole human corneas or the outer limbal rims were cut into six pieces (1). A corneal brush was used to remove the epithelium in the direction indicated avoiding damage to the limbus where the stem cells reside (2). Following culture the epithelium migrated across the tissue to heal the wound (3).

were stained with haematoxylin. The sections were then dehydrated and mounted.

2.4. In situ hybridisation

Based on histological data, specimens from the nine tissue series representing the original unwounded tissue, the wound, the re-epithelialisation process and the healed tissue were analysed. The production and specificity of the antisense human MMP-1, -3, -10 and TIMP-1 RNA probes have been described previously (Saarialho-Kere et al., 1992, 1994). Sections were hybridised with ^{35}S-labelled sense RNA from a bovine tropoelastin cDNA as a control for non-specific binding. The validity of this probe as a negative control has been confirmed by northern blotting (Prosser et al., 1989) and by in situ hybridisation (Saarialho-Kere et al., 1992).

In situ hybridisation was carried out as previously described (Prosser et al., 1989). Briefly, samples were pre-treated with proteinase K (1 μg ml^{-1}) then washed in 0·1 M triethanolamine buffer containing 0·25% acetic anhydride. The sections were covered with 35 μl of hybridisation buffer containing 2·5 × 104 per μl of 35S-labelled antisense or sense (negative control) RNA probe, and incubated at 50–60°C for 18 hr in a humidified chamber. After hybridisation, the slides were washed under stringent conditions, including treatment with RNaseA to remove unhybridised probe. Following 15–45 days of autoradiography, the photographic emulsion was developed, and the slides were stained with haematoxylin and eosin. Cutaneous wounds known to express MMP-1, -3, -10 and TIMP-1 were used as positive controls and each sample with several sections was hybridised in two different experiments. Sections were assessed with both dark-field and bright-field microscopy by two independent observers.

2.5. Antibodies

MMP-9 protein was detected with a monoclonal antibody (GE213, Diabor, Oulu, Finland) (Rechardt et al., 2000). Laminin-5 (LN-5) producing cells were identified with polyclonal rabbit antibodies against the γ-2 chain of LN-5 (Pyke et al., 1994; Airola et al., 1997). A mouse monoclonal antibody to cytokeratin 3 (AE5, ICN Pharmaceuticals, Ltd., Basingstoke, UK) was used to demonstrate that the migrating epithelial cells were of corneal and not conjunctival origin.

2.6. Immunohistochemistry

Immunohistochemistry on sections serial to those used for in situ hybridisation was performed. All sections were pre-treated with 10 mg ml^{-1} trypsin for 30 min at room temperature. MMP-9 and LN-5 were detected using the avidin-biotin-peroxidase technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) as previously described (Saarialho-Kere et al., 1993). Cytokeratin 3 was detected using the streptavidin–biotin alkaline phosphatase complex (Dako) as previously described (Webster et al., 1999; Daniels and Khaw, 2000). Diaminobenzidine was used as the chromogenic substrate and Harris Heamatoxylin for counterstaining. The antibody dilutions were; MMP-9 1:200 and LN-5 1:500 and 5 μg ml^{-1} of cytokeratin 3. Controls were performed with mouse immunoglobulins or with rabbit preimmune serum.

3. Results

3.1. Wounded tissue healing

Several techniques to wound the corneal tissue pieces were attempted including scraping away the epithelium using a scalpel or biopsy punch to remove tissue. The method chosen for this study was the most reproducible and involved using a corneal brush (Algerbrush) to remove epithelium. (Daniels et al., 2003b) Care was taken not to pass the brush over the limbus as illustrated in Fig. 1.

Of 11 corneas/limbal rims wounded and cultured, two were discarded as no healing was observed. Owing to

the differences in sizes between the limbal and whole corneal tissue samples, the times taken to achieve healing differed slightly. Time to achieve re-epithelialisation of the anterior stroma, defined by the presence of an intact monolayer or multi-layered epithelium, ranged from 3 to 7 days post-wounding for corneas and 2–5 days for limbal rim material. For direct comparison of data, results obtained from four corneas are shown throughout the figures. Interestingly, despite the longer storage period of limbal rims before use, the profiles of each parameter studied did not appear to vary between limbal and corneal tissue.

Immunohistochemical staining for cytokeratin 3, a marker for cells of corneal origin (Schermer et al., 1986), confirmed that re-epithelialisation most likely occurred with cells of corneal origin (data not shown).

Corneal epithelium rests upon a BM. The PAS reaction, which detects carbohydrates and glycoproteins, is one of the most reliable methods for demonstrating the corneal epithelial BM (Apple and Rabb, 1985). Due to the difficulty in obtaining commercially available antibodies that will work on paraffin sections, the PAS reaction was used as an indicator of BM deposition. In Fig. 2(A) the continuous BM of the unwounded tissue is indicated by the solid arrow. Directly beneath the BM is an acellular modified layer of the anterior corneal stroma known as Bowman's layer (BWL, indicated by the dotted arrow in Fig. 2(A)), the main component of which is type I collagen. Type VII collagen fibrils are also found in this layer. Using connective tissue stains, BWL is almost histologically identical to the rest of the corneal stroma (Apple and Rabb, 1985), yet its function remains unclear. Following application of the corneal brush epithelial cells were removed and the substrates detected by the PAS reaction were disrupted in all of the tissue series (Fig. 2(B)). It has previously shown that BM disruption only occurs some time after the creation of large debridement wounds in the mouse (Sta Iglesia and Stepp, 2000), indicating that our model may represent more extensive BM damage. In all but one of the tissue sections wounded

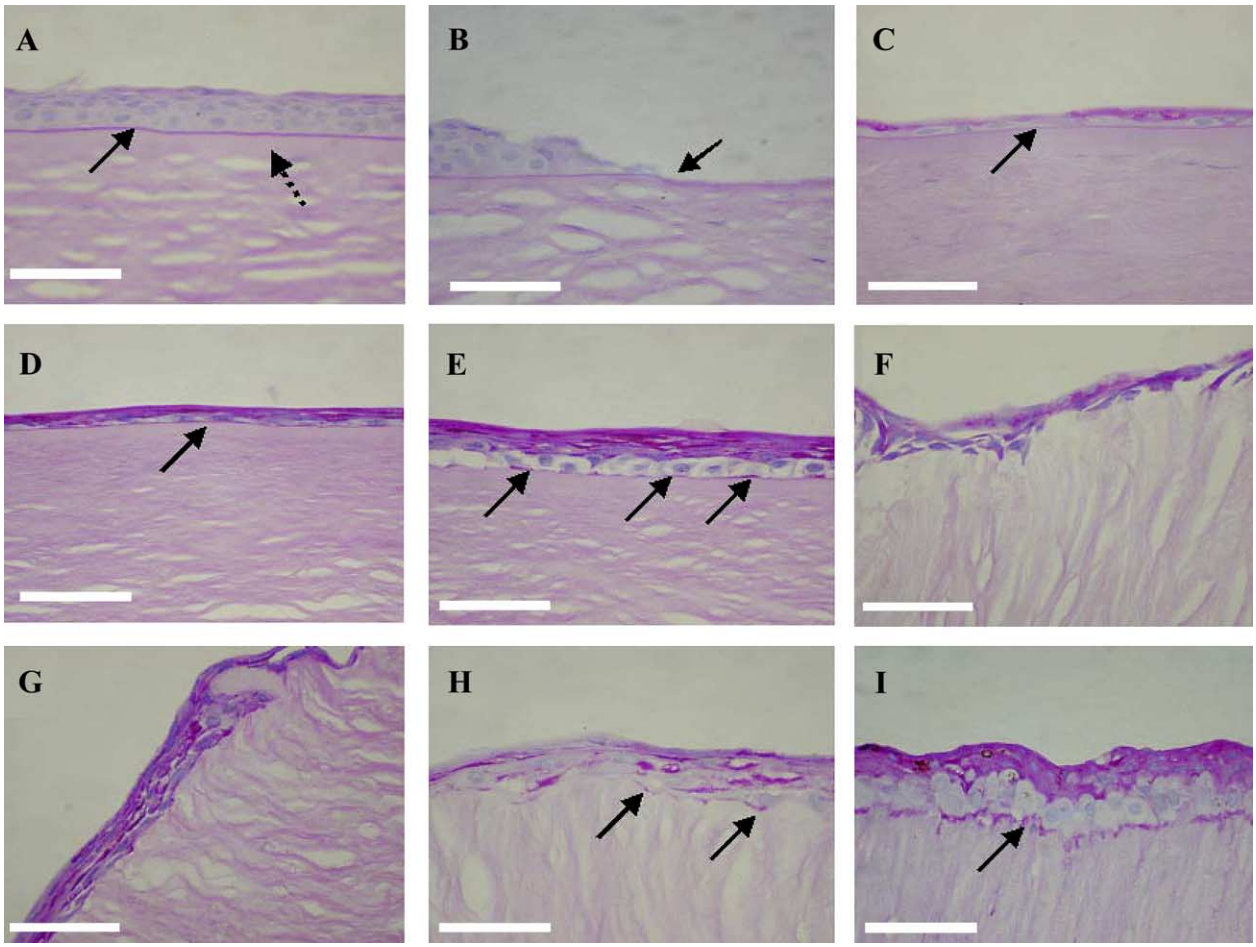


Fig. 2. The presence of components of the corneal epithelial basement membrane is indicated by the PAS reaction. The basement membrane is indicated by the solid arrow. The acellular Bowman's layer, beneath the basement membrane, is shown by the dotted arrow (A). Following wounding, the basement membrane is disrupted (B). As an epithelial monolayer forms over Bowman's layer (C) and early differentiation (D) begins a faint positive PAS reaction is seen beneath the basal cells. As multi-layered epithelium develops the intensity of the PAS reaction increased (E). No PAS reaction was detected by day 3 beneath several (F) or multiple (G) layers of epithelial cells on the cut edge of the tissue. By 7 days post-injury a positive PAS reaction was detected beneath the multi-layered epithelial cells on the cut edge of the tissue (H and I). The scale bar represents 100 μm.

BWL remained intact. Immediately post-wounding BWL appeared slightly thinner than the neighbouring unwounded region (arrowed in Fig. 2(B)). However, post-culture it returned to the normal depth of approximately 10 μm. The epithelial cells then started to migrate from the non-wounded area after 2–3 days until a single intact monolayer could be seen covering the surface of the wound (Fig. 2(C)). The epithelial cells appeared to be synthesising components of a new BM indicated by the faint PAS reaction beneath the cells (arrowed in Fig. 2(C)). However, this reaction was not as strong as that seen in the unwounded tissue. As the epithelium developed into several layers (Fig. 2(D)), the PAS reaction remained a similar intensity to the monolayers. However, by 7 days when more epithelial cell layers (including what appeared to be squames) had developed, the intensity of the PAS reaction started to increase (arrowed in Fig. 2(E)), suggesting that deposition of BM components was continuing. Although the morphology of the repairing epithelium at 7 days was not identical to that of the unwounded tissue, basal, wing and squamous cells were detectable. In this study the corneas were removed from the eye therefore during re-epithelialisation, epithelial cells continued to migrate around the cut edge of the stroma. Marked differences in the PAS reaction were detected depending on whether the epithelial cells had migrated over BWL or not. No PAS reaction was detected by day 3 beneath several (Fig. 2(F)) or multiple (Fig. 2(G)) layers of epithelial cells on the cut edge of the tissue. This was in contrast to the faint PAS reaction beneath the monolayer of cells in the presence of BWL (Fig. 2(C)). The final appearance of the epithelium at 7 days at the cut edge of the stroma was morphologically different to that of the repaired epithelium covering the wound, yet a positive PAS reaction beneath the epithelium was evident (Fig. 2(E) and (H)). This suggests that the epithelial cells could still deposit BM components despite the absence of BWL.

3.2. Identification of MMPs and TIMP-1

The percentage of the total number of tissues examined at each stage of re-epithelialisation i.e. epithelial cell migration, formation of an intact monolayer and development of multi-layered epithelium expressing MMPs or TIMP-1 is shown in Table 1. In situ hybridisation was used to detect mRNA for MMPs 1, 3, 10 and TIMP-1. Immunohistochemistry was used to detect MMP-9 protein.

In all of the tissues examined, MMP-1 was expressed during epithelial cell migration over BWL and around the cut edge of the tissue (Fig. 3(A)). Since the BM was disrupted, the cells were likely to be migrating over substrates including type I collagen, the most abundant component of BWL and also of corneal stroma over which the cells moved at the cut edge. The development of an intact monolayer of epithelial cells over Bowman's membrane or the cut edge of the stroma coincided with cessation of MMP-1 expression in these cells in all samples.

Table 1
Percentage of total samples positive for MMP/TIMP

Stage of re-epithelialisation	MMP-1		MMP-3		MMP-9		MMP-10		TIMP-1	
	E	S	E	S	E	S	E	S	E	S
Migration	100	85	0	100	25	0	100	14	20	100
Intact monolayer	0	100	0	75	100	0	50	20	50	100
Multiple layers	0	0	0	75	100	0	50	20	50	50

The total number of samples, expressed as a percentage, positive for MMP/TIMP are shown in the table. MMP-1, -3, -10 and TIMP-1 mRNA was detected by in situ hybridisation while immunohistochemistry was used to detect MMP-9 protein. For the purposes of interpretation the process of re-epithelialisation is divided into migration, development of an intact monolayer and the development of multiple layers. E and S represent epithelial and stromal cells, respectively.

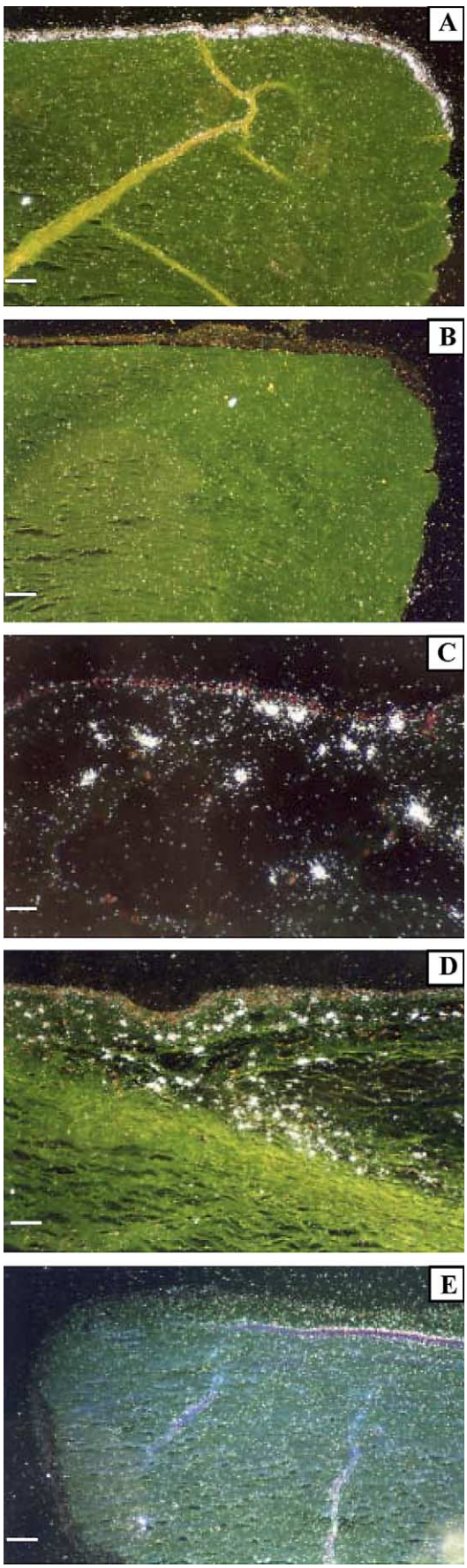
No correlation between a positive or negative PAS reaction beneath the epithelial cells and down-regulation of MMP-1 expression by epithelial cells was detected.

MMP-1 was expressed by fibroblast-like stromal cells beneath the migrating epithelial cells and remained present in the stroma upon formation of an epithelial monolayer around the tissue (Fig. 3(C)). MMP-1 expression continued in the stroma as several layers of epithelial cells started to develop (Fig. 3(D)). Development of a multi-layered epithelium over BWL and around the cut edge of the tissue coincided with the absence of MMP-1 in the stroma of all of the samples (Fig. 3(E)). No signal was detected in the sense control (Fig. 3(B)).

MMP-3 was not detected in epithelial cells at any stage of healing (Fig. 4). In contrast, MMP-3 was always found in the fibroblast-like stromal cells beneath epithelial cells migrating over BWL, and deeper in the stroma (Fig. 4(A)) and was still present at epithelial monolayer development over BWL in most cases (Fig. 4(C)). As for MMP-1, MMP-3 was also switched off in stromal cells in the presence of multi-layered epithelium surrounding the tissue in 25% of the samples (Fig. 4(D)) but remained present in isolated areas in the others (Fig. 4(E)). No signal was detected in the sense control (Fig. 4(B)).

The identification of MMP-10 expression by corneal epithelial cells during wound repair is a novel finding. MMP-10 was expressed by epithelial cells migrating over BWL and by the basal cells of epithelium behind the leading edge (Fig. 5(A)). Unlike MMP-1, MMP-10 remained in the epithelial cells when a monolayer had developed across BWL and the cut edge of the stroma in half of the tissues examined (Fig. 5(C)). However, upon formation of multi-layered epithelium surrounding the tissue, MMP-10 was switched off in 50% of the samples tested (Fig. 5(D)). No signal was detected in the stromal cells or sense control (Fig. 5(B)).

TIMP-1 was associated with fibroblast-like stromal cells during epithelial cell migration over BWL (Fig. 6(A)). TIMP-1 remained in the fibroblast-like stromal cells



beneath the newly formed intact epithelial cell monolayer and deeper in the stroma (Fig. 6(C)). Upon development of multi-layered epithelium surrounding the tissue, less TIMP-1 expression was detected in the stromal cells (Fig. 6(D)). No signal was detected in the sense control (Fig. 6(B)).

MMP-9 was associated with epithelial cell migration as the cells came into contact with type I collagen at the cut end of the corneal tissue (Fig. 7(A)). MMP-9 was not detected in cells in contact with BWL, the end of which is indicated by the dotted arrow (Fig. 7(A)). However, MMP-9 remained present in the epithelial basal cells of multi-layered epithelium across the cut edge of the stroma (Fig. 7(C)). MMP-9 was never found in stromal cells. No immunostaining was detected in the negative control samples (Fig. 7(B)).

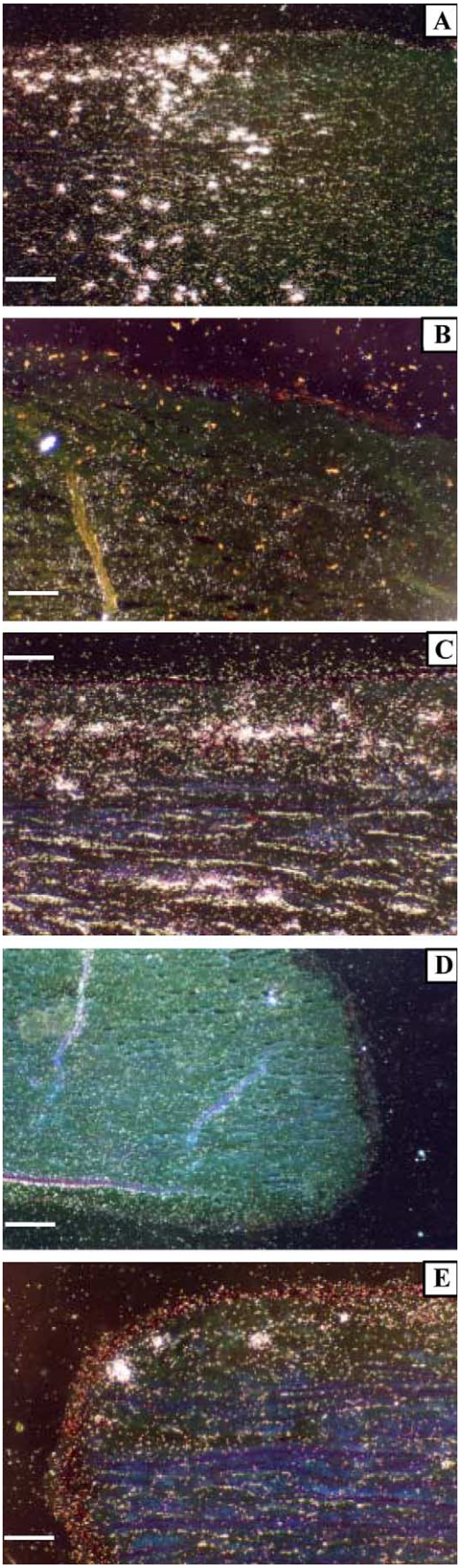
LN-5 protein, a component of the corneal BM was detected by immunohistochemistry inside epithelial cells in all of the samples as they migrated over BWL and around the cut edge of the stroma (Fig. 8(A)). Upon formation of a multi-layered epithelium at the cut stromal end LN-5 was found to be still detectable within the basal cells and now also deposited extracellularly in the underlying matrix (Fig. 8(C)). No immunostaining was detected in the negative control (Fig. 8(B)).

4. Discussion

Matrix metalloproteinases are a very potent family of enzymes capable of devastating tissue destruction in various organs when regulation mechanisms fail. The potential clinical use of specific synthetic inhibitors or antisense oligonucleotides to MMPs may soon be possible. In order to do this safely it is essential to identify the MMPs involved in normal human wound healing and to understand their individual modes of action.

This study demonstrates temporal and spatial expression of MMPs following wounding of human corneal tissue, contrasting many previous studies in which only one or two MMPs have been studied in animal models (Sivak and Fini, 2002). Following wounding of the epithelium with a corneal brush, re-epithelialisation of the ex vivo tissue pieces was achievable in the culture conditions used. As serial corneal biopsies cannot be harvested from living human subjects

Fig. 3. The profile of MMP-1 expression during healing of the wounded human corneal tissue is shown by in situ hybridisation. The silver grains indicating a positive result are seen in white. Epithelial cells migrating over the wound and around the cut edge of the tissue expressed MMP-1 (A). Once the tissue was surrounded by an intact epithelial monolayer, MMP-1 was switched off in the epithelial cells (C). MMP-1 was also expressed by fibroblast-like stromal cells beneath the leading epithelial edge remained present in the fibroblast-like stromal cells beneath the epithelial monolayer (C). MMP-1 persisted in the stroma as several layers of epithelial cells started to develop (D). However, upon formation of a multi-layered epithelium around the tissue, MMP-1 was switched off in the stroma (E). No signal was detected with the sense control (B). The scale bar represents 100 μm.



without compromising sight, the ex vivo tissue culture system appeared to be a reasonable method for assessing MMPs in human tissue. Human donor corneas can be routinely cultured at 37°C for up to 1 month before transplantation, maintaining the integrity of the epithelium and endothelium (Crewe and Armitage, 2001). Therefore the study tissues were used well within an acceptable clinical time frame for tissue viability and integrity.

The role of BWL during human corneal wound healing is unclear. This layer is composed of a molecules including type I collagen, the most abundant component (Ljubimov et al., 1996). In our system BWL appeared to provide a favourable substrate for the deposition, by epithelial cells, of products identified by the PAS reaction. Fundamental differences exist between the organisation of basement membrane components in different parts of the cornea (Ljubimov et al., 1995, 1998). Deposition of BM components could represent an attempt by the epithelial cells to synthesise new BM following injury. For example continuous areas of PAS positivity, albeit faint, were detected beneath monolayers of epithelial cells on BWL. This process was delayed at the cut edge of the stroma where multiple layers of epithelial cells needed to be developed before a PAS reaction was detectable. LN-5 is a component of the corneal BM (Ljubimov et al., 1995). Excessive amounts of LN-5 were deposited by the cells at the cut edge compared to the more discreet intracellular expression by cells on BWL. This could indicate the cells were trying to produce a new BM over the cut edge but that the underlying substrate was unfavourable and so LN-5 synthesis and deposition was not properly regulated. The presence of BWL was not essential for epithelial cells to form multi-layered epithelium; however, differences in morphology occurred which may indicate a role for this acellular layer in the correct organisation and regulation of human corneal epithelial cell differentiation.

Leading keratinocytes migrating upon exposed dermal type I collagen deposit precursor LN-5 mediating motility. Cleavage of the precursor molecule to mature LN-5, required for type I hemidesmosome assembly, may signal the epithelial cells to become quiescent and form integrated tissue (Nguyen et al., 2000). Our anti-LN-5 antibody could not distinguish between precursor and mature protein. However, intracellular staining in areas of BM damage and around the cut edge of the stroma indicated that the migrating corneal epithelial cells deposited LN-5. MMPs

Fig. 4. The profile of MMP-3 expression during healing of human corneal tissue as assessed by in situ hybridisation. MMP-3 was not detected in epithelial cells. During healing fibroblast-like stromal cells expressed MMP-3 beneath the migrating epithelial cells and deep into the stroma (A). MMP-3 was present in the stroma when an epithelial monolayer surrounded the tissue (C). Upon formation of multi-layered epithelium MMP-3 was switched off in the stroma of one sample (D) but remained present in isolated areas in the stroma (E). No signal was detected with the sense control (B). The scale bar represents 100 μm.

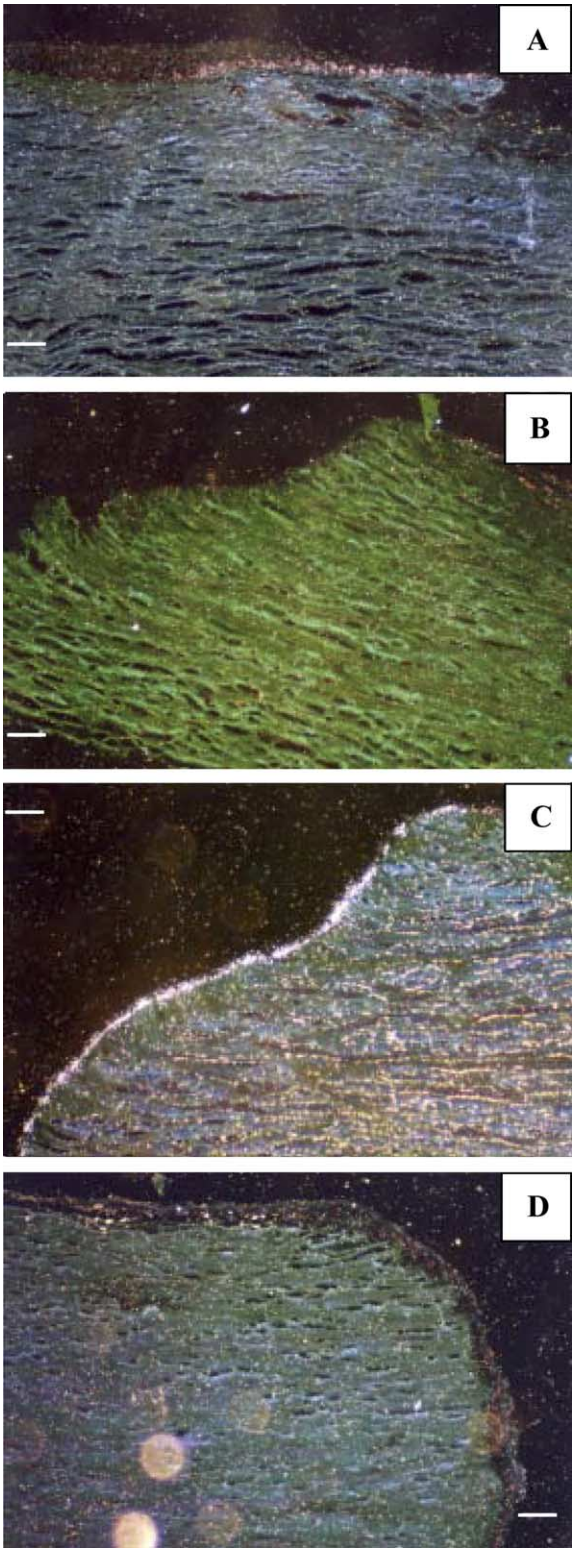


Fig. 5. The profile of MMP-10 expression during healing of the human corneal tissue is shown by in situ hybridisation. Migrating epithelial cells expressed MMP-10 (A) that remained in the cells when they surrounded the tissue with a monolayer (C). Upon formation of multi-layered epithelium, MMP-10 remained in the basal cells in some tissue, however, when the tissue appeared to be surrounded by multi-layered epithelium MMP-10 was switched off in the epithelial cells (D). No signal was detected in the sense control (B). The scale bar represents 100 μm.

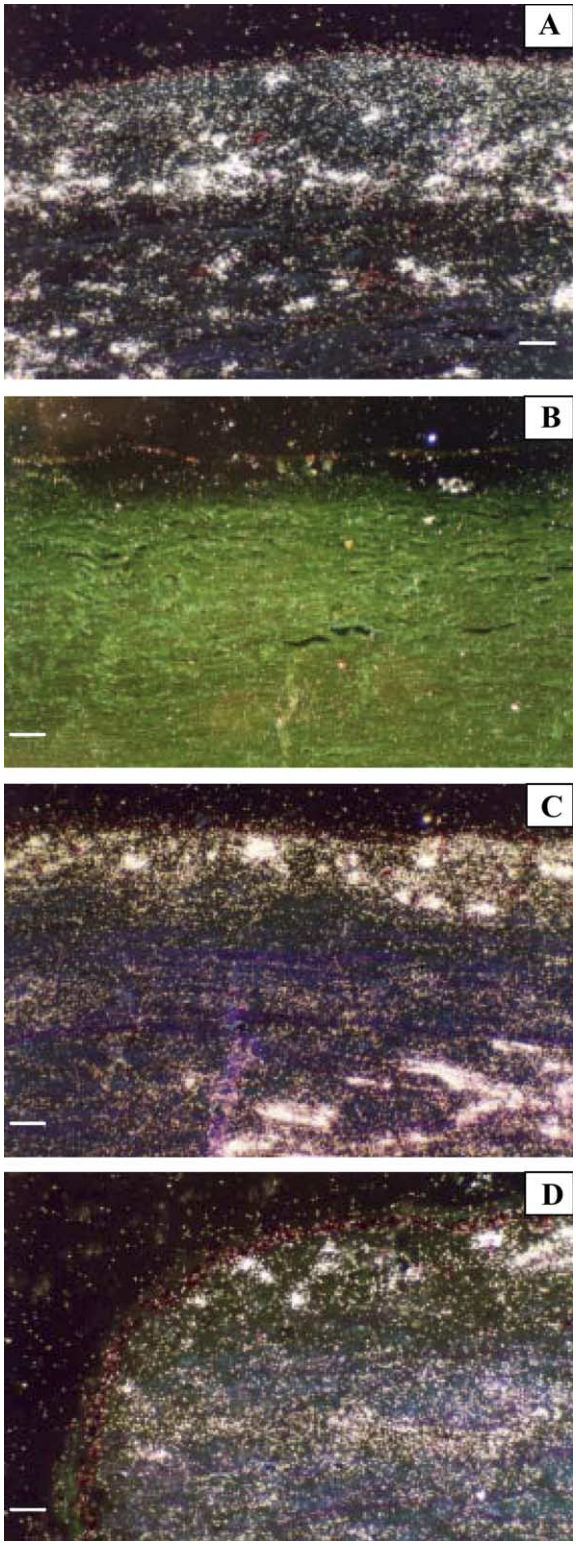


Fig. 6. The profile of TIMP-1 expression, which was mainly associated with the stroma during human corneal wound healing, is shown by in situ hybridisation. TIMP-1 was associated with fibroblast-like stromal cells during epithelial cell migration (A). TIMP-1 remained in the fibroblast-like stromal cells beneath the newly formed intact epithelial cell monolayer and deeper in the stroma (C). Upon development of multi-layered epithelium less TIMP-1 expression was detected in the stromal cells (D). No signal was detected with the sense control (B). The scale bar represents 100 μm.

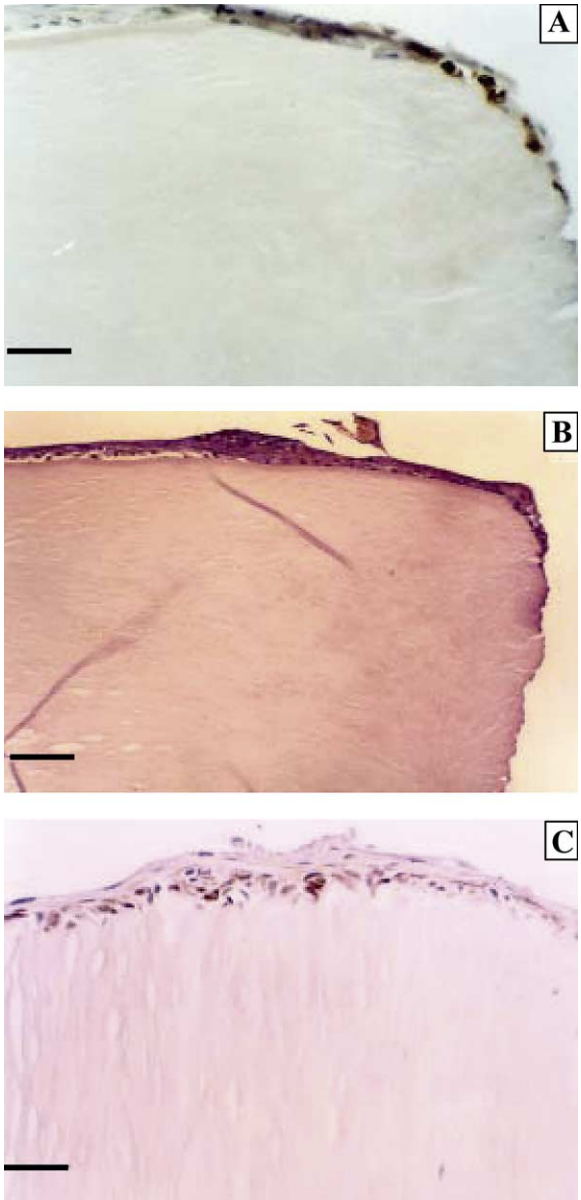


Fig. 7. The profile of MMP-9 protein production, during healing of the human corneal tissue is shown by immunohistochemistry. The dark brown cells are positive. The epithelial cells migrating around the cut edge of the tissue produced MMP-9 (A). More often MMP-9 was associated with the basal cells of multi-layered epithelium of healed tissue (C). No immunostaining was detected in the control tissue (B). The scale bar represents 100 μm.

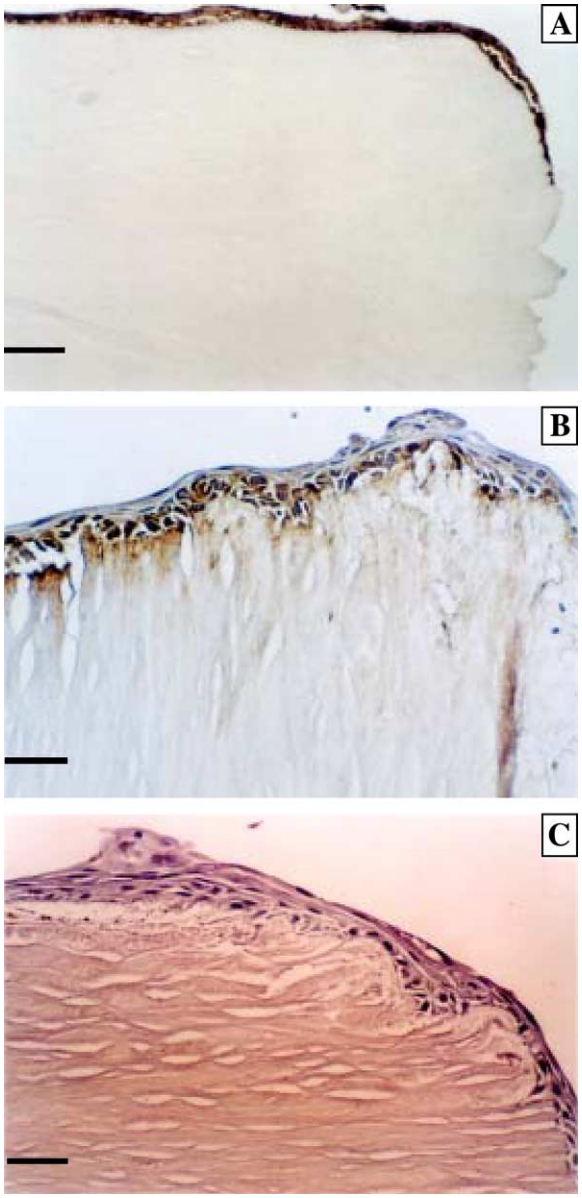


Fig. 8. The profile of LN-5 protein production, during healing of the human corneal tissue is shown by immunohistochemistry. The dark brown cells are positive. LN-5 was found in association with cells migrating over bare stroma (A) and behind the leading front. LN-5 staining remained in the basal cells upon formation of multi-layered epithelium at the end of healing (B). No staining detected in the control tissue (C). The scale bar represents 100 μm.

have a potential role in LN-5 processing in the cornea. In support of this theory, addition of exogenous (cleaved) LN-5 to cultured corneal epithelial cells promoted cell adhesion, spreading and formation of hemidesmosomes whereas endogenous (non-cleaved) secreted LN-5 promoted cell migration (Ebihara et al., 2000). A role for the membrane type MMP-2 and MT1-MMP have already been suggested as a trigger for migration upon LN-5 (Giannelli et al., 1997; Koshikawa et al., 2000).

MMPs 1 and 10 were up-regulated by epithelial cells migrating over BWL and the cut edges of the tissues. Localisation of MMP-1 expression strongly suggests that corneal epithelial cells use MMP-1 to migrate upon type I collagen and that down-regulation of MMP-1 occurs as a result of cell–cell contact inhibition, in accordance with previous findings in human skin (Inoue et al., 1995), regardless of the source of the underlying type I collagen substrate i.e. BWL or cut stroma. We have recently shown that human corneal epithelial cells need MMP-1 to migrate on type I collagen in vitro. (Daniels et al., 2003b). This concurs with previous in vitro studies on cultured cutaneous keratinocytes (Pilcher et al., 1997).

MMP-3 is absent in unwounded human whole corneas (Saghizadeh et al., 2001) and was not found in repairing corneal epithelial cell either in accordance with previous reports in the rabbit cornea, human tears and cultured human corneal epithelial cells. (Fini and Girard, 1990; Sobrin et al., 2000). These results are in contrast to the data found during human cutaneous wound repair (Vaalamo et al., 1996). Our results on MMP-10 (stromelysin-2) present novel findings not previously reported in any corneal wound repair study of human or animal origin. However, while this study was in progress, Saghizadeh et al. (2001) demonstrated that MMP-10 is overexpressed in the corneal epithelium of patients with diabetic retinopathy possibly contributing to the BM zone protein changes. Type IV collagen, laminin-1, fibronectin and nidogen are components of the corneal BM (Ljubimov et al., 1995; Fukuda et al., 1999) and also an in vitro substrate for MMP-10 (Birkedal-Hansen et al., 1993). The PAS reaction in our samples indicated BM disruption occurred during injury. MMP-10 may facilitate cell migration during re-epithelialisation, analogously to what is seen in cutaneous suction blisters (Rechardt et al., 2000), over remnants of any type IV collagen left behind on the wound. However, the persistence of MMP-10 during migration over BWL and the cut edge of the stroma lacking type IV collagen indicates a further role for MMP-10. In normally healing skin MMP-3 is produced by pre-migratory keratinocytes resting on BM (Vaalamo et al., 1996) perhaps to break cell–cell contacts by E-cadherin cleavage (Lochter et al., 1997). As corneal epithelial cells did not produce MMP-3, MMP-10 may replace this potential role in the cornea. Growth factors such as tumour necrosis factor alpha, epidermal growth factor and transforming growth factor beta may induce MMP-10 in corneal epithelial cells as they do in cutaneous keratinocyte cultures (Rechardt et al., 2000). MMP-10 was switched off in the basal epithelial cells of half of the samples examined once a multi-layered epithelium had formed over BWL and around the cut edge, perhaps indicating an end to a role in BM re-assembly and re-modelling in these samples.

In contrast to previous data from the rat where MMP-9 was found to localise exclusively to migrating epithelial cells (Ye and Azar, 1998) MMP-9 remained present even upon formation of multi-layered epithelium and the deposition of PAS reaction positive material at the cut edge. This indicates a role for MMP-9 in human corneal BM re-modelling as previously suggested in the rabbit (Matsubara et al., 1991; Fini et al., 1992, 1998). Re-epithelialisation of the cornea, skin and trachea in MMP-9 knock-out mice is faster than in wild type animals, suggesting that MMP-9 inhibits the rate of wound closure by altering the rate of epithelial cell proliferation (Mohan et al., 2002). Furthermore, MMP-9 knock-out cells did not appear to be able to clear provisional matrix from wounds, again supporting a role for MMP-9 in BM re-assembly (Mohan et al., 2002). MMP-9 may also play an as yet undefined role in epithelial cell differentiation. We have previously demonstrated up-regulation of MMP-9 during development of mature multi-layered corneal epithelium in vitro (Daniels et al., 2000).

In the early stages of healing MMPs 1 and 3 are switched on in stromal cells together with TIMP-1. Fibroblast-like stromal cells expressed MMP-1 including the area beneath the repairing epithelium. This location suggests these cells could use MMP-1 for recruitment to the wound area and or to re-model damaged and newly deposited collagen I. It is thought that many cell types use MMPs to migrate through extracellular matrix including smooth muscle cells (Bendeck et al., 1994; Pauly et al., 1994), retinal growth cones (Sheffield et al., 1994), and intestinal mucosal mesenchymal cells (Pender et al., 2000). We have recently demonstrated that human ocular fibroblasts require MMPs to re-organise and contract collagen matrix in vitro (Daniels et al., 2003a).

MMP-3 (stromelysin-1) was expressed by fibroblast-like stromal cells beneath migrating epithelial cells, newly formed multi-layered epithelium and deeper in the stroma, suggesting roles in BM assembly and stromal re-modelling. Upon establishment of multi-layered epithelium MMP-3 was switched off in the stroma in one quarter of the samples. We previously found that cultured multi-layered epithelium suppresses fibroblast wound healing activities including migration and matrix contraction (Daniels and Khaw, 2000). Perhaps re-establishment of a mature epithelium is one of the signals for the cessation of wound healing, including MMP production. Clinical evidence of persistent corneal epithelial defects resulting in ulceration as the tissue re-modelling process continues uncontrolled supports this theory (Jenkins et al., 1979; Waring and Rodrigues, 1987; Akpek et al., 1997).

The expression of TIMP-1 throughout corneal wound healing was not surprising and agrees with previous findings (Vaalamo et al., 1996). Its location was also similar to that previously found in the rat (Ye and Azar, 1998). TIMP and MMP expression must be balanced in order to prevent excess matrix deposition or degradation (Birkedal-Hansen, 1995). The presence of TIMP-1 beneath the epithelial cells may have served to restrict MMP activity to the migratory front.

In conclusion, our novel findings demonstrate that MMP-1 and -10 are expressed by epithelial cells migrating over BWL. Human corneal repair is also characterised by temporally and spatially tightly controlled expression of MMP-3, MMP-9 and TIMP-1.

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